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# FMRFamide signaling promotes stress-induced sleep in *Drosophila*

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ABSTRACT

Enhanced sleep in response to cellular stress is a conserved adaptive behavior across multiple species, but the mechanism of this process is poorly understood. *Drosophila melanogaster* increases sleep following exposure to septic or aseptic injury, and *Caenorhabditis elegans* displays sleep-like quiescence following exposure to high temperatures that stress cells. We show here that, similar to *C. elegans*, *Drosophila* responds to heat stress with an increase in sleep. In contrast to *Drosophila* infection-induced sleep, heat-induced sleep is not sensitive to the time-of-day of the heat pulse. Moreover, the sleep response to heat stress does not require *Relish*, the NFκB transcription factor that is necessary for infection-induced sleep, indicating that sleep is induced by multiple mechanisms from different stress modalities. We identify a sleep-regulating role for a signaling pathway involving FMRFamide neuropeptides and their receptor FR. Animals mutant for either FMRFamide or for the FMRFamide receptor (FR) have a reduced recovery sleep in response to heat stress. *FR* mutants, in addition, show reduced sleep responses following infection with *Serratia marcescens*, and succumb to infection at a faster rate than wild-type controls. Together, these findings support the hypothesis that FMRFamide and its receptor promote an adaptive increase in sleep following stress. Because an FMRFamide-like neuropeptide plays a similar role in *C. elegans*, we propose that FRMFamide neuropeptide signaling is an ancient regulator of recovery sleep which occurs in response to cellular stress.



## 1. Introduction

An increase in sleep following an immune challenge is common to many species (reviewed in (Imeri and Opp, 2009)), including humans (Mullington et al., 2000) and *Drosophila* (Kuo et al., 2010). Similar to mammals, the *Drosophila* acute sleep response to infection is dependent on the time of day of inoculation, and requires an NFκB transcription factor, *Relish* (Kuo et al., 2010). A recent study shows that bacterial toxins, as well as other stressors, such as osmotic shock or heat shock, induce sleep-like quiescence in adult *Caenorhabditis elegans* nematodes (Hill et al., 2014). Together, these findings indicate that sleep is a conserved response to stressful stimuli. Importantly, the sleep response promotes survival (Hill et al., 2014; Kuo and Williams, 2014a), indicating that it is adaptive and beneficial to the animal.

Many components of the mammalian innate immune response, particularly pro-inflammatory cytokines, exert sleep-promoting effects likely through actions in hypothalamic nuclei (Obal and Krueger, 2003). In flies, expression of *Relish* in the fat body, which is a major site of immune response signaling, is necessary for the sleep promoting effects of aseptic injury and immune challenge (Kuo et al., 2010) and has a role in daily night-time sleep regulation (Williams et al., 2007). We have recently demonstrated that altering neuronal excitability in the mushroom body to manipulate sleep (Joiner et al., 2006) influences survival outcome during bacterial infections (Kuo and Williams, 2014b). Thus, communication from peripheral tissue to the brain is likely a key mechanism that underlies the injury/infection-induced sleep response in *Drosophila*. However, the signaling molecules and neuronal circuitry that underlie these processes are unknown.

In *C. elegans*, the neurosecretory ALA neuron promotes the sleep-like quiescence in response to cellular stress (Hill et al., 2014). In response to heat stress, the ALA neuron depolarizes and releases neuropeptides encoded for by the gene *flp-13* (Nelson et al., 2014). FLP-13 peptides are similar to *Drosophila* Phe-Met-Arg-Phe-amide (FMRFa) peptides. FMRFa is a member of the FMRFa related peptide family (FaRP), which in addition to

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FMRFa, include dromyosuppressin, drosulfakinin, neuropeptide F (NPF), and short neuropeptide F (sNPF) (Nassel and Winther, 2010). Both NPF and sNPF have sleep-regulating roles (Chen et al., 2013; He et al., 2013; Shang et al., 2013). Each of these peptide genes encodes for one or more peptides with *Fmrfa* encoding for eight (Schneider and Taghert, 1990). *Fmrfa* is expressed in the central nervous system (CNS) as well as in thoracic neurosecretory cells (Nassel and Winther, 2010). The function of *fmrfa* is not fully understood, but has been reported to be important for escape responses in larval (Klose et al., 2010) and adult flies (Kiss et al., 2013). The FMRFa receptor (FR; CG2114) has varying affinity for the different FMRFa peptides and is also capable of binding Dromysosuppressin (Cazzamali and Grimmelikhuijzen, 2002; Johnson et al., 2003; Meeusen et al., 2002). FR is related to the neurotensin/thyrotropin-releasing factor receptor family in mammals (Johnson et al., 2003). Based on the fact that FLP-13 peptides promote sleep-like quiescence in response to stress in *C. elegans*, we tested the hypothesis that FMRFa and its corresponding receptor promote the stress-induced sleep response in *Drosophila*.

## 2. Material and methods

### 2.1. Fly stocks

Flies were grown on standard dextrose-cornmeal media. Control strains included *Canton-Special* (*CS*), $w^{1118}$, and *yellow*, *white* (*y*,*w*). Other strains were obtained from the Bloomington Drosophila Stock Center (Indiana University), and include *Mi{ET1}* $FMRFaR^{MB04659}$, $FMRFaR^{JF01879}$, *Df(3L)BSC428*, and *P{SUPor P}* $FMRFa^{KG01300}$. $Relish^{E20}$ mutants (Hedengren et al., 1999) were isogenized to *CS* as previously described (Williams et al., 2007).

### 2.2. Behavioral assays

Locomotor activity and sleep were measured as described previously (Kuo et al., 2012). Briefly, female flies that were 1–3 days of age were loaded into glass activity tubes (65 mm in length) containing 5% sucrose, 2% agar medium at one end and plugged with cotton yarn at the other end. Glass tubes were loaded into Drosophila Activity Monitors (DAM2, Trikinetics, Inc., Waltham, MA), and activity was recorded for up to 7–10 days. Monitors were placed in incubators kept at 25 °C in 12 h:12 h light:dark cycles or constant light and 50% humidity. In this assay, activity counts correspond to breaks of an IR beam that bisects the tube. Sleep is defined as an activity count of zero for a minimum of 5 consecutive minutes (Huber et al., 2004). Data were processed using custom software, Insomniac 3 (written in MSCV6, by Thomas Coradetti).

Flies were exposed to heat shock by transferring monitors to an incubator in the same light phase, but set to a temperature of 37 °C. After 1 h, flies were returned to the 25 °C incubator. Monitors containing handled control flies were briefly removed from the 25 °C incubator, but immediately returned without exposing to a heat pulse. Sleep responses were quantified as described previously (Kuo et al., 2010). Briefly, differences between minutes of sleep in a given time increment following treatment (heat pulse or infection) and the equivalent baseline time increment were calculated. This difference was subtracted from an average difference for

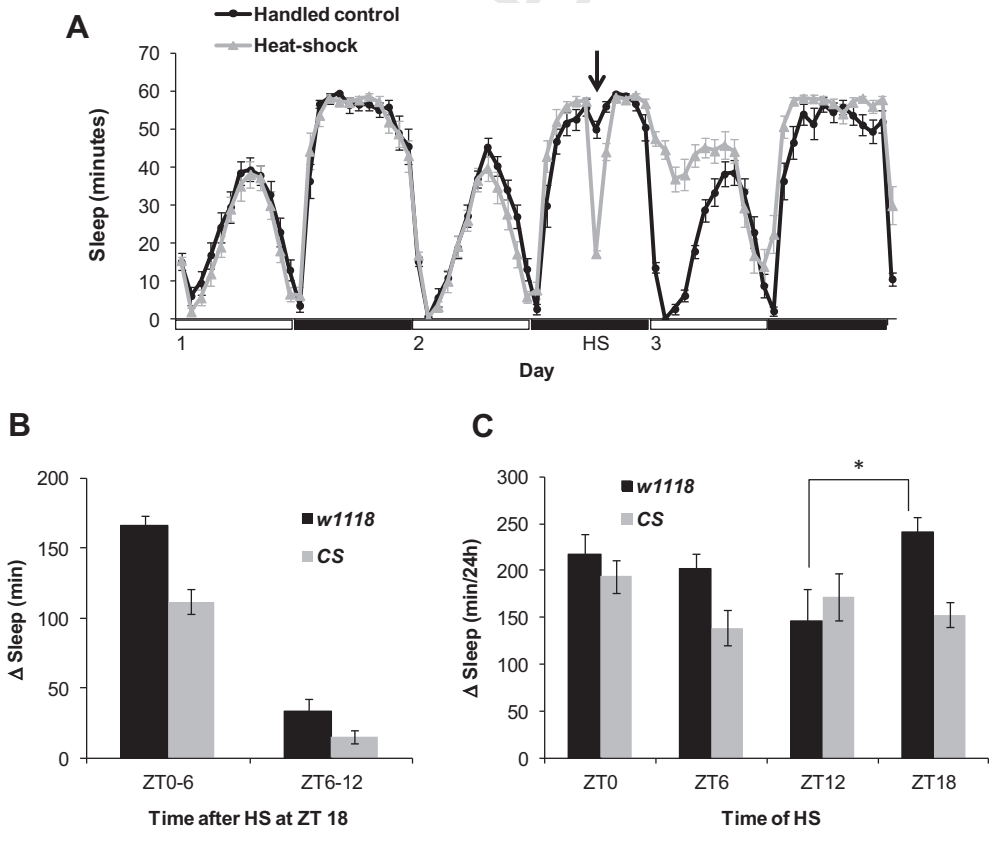


**Fig. 1.** Heat stress induces daytime sleep. (A) Mean ± SEM sleep (in minutes) per hour is plotted for $w^{1118}$ flies exposed to a 1 h 37 °C heat pulse starting at ZT 18 (arrow, "HS"). Horizontal bars along the *x*-axis indicate light:dark phases; $n = 16$ flies each for handled control and heat shocked groups. (B) Mean ± SEM net change in sleep (minutes/6 h) is shown at indicated times for $w^{1118}$ and *CS* flies following HS at ZT 18 ($n = 78$ and 32 $w^{1118}$ and *CS* flies, respectively). (C) Mean ± SEM net change in sleep (minutes/24 h) is shown for $w^{1118}$ and *CS* flies subjected to HS at indicated times of day ($n = 32$–78 flies for each group; $^{*}p < 0.01$ Tukey's post-hoc; see main text for details).

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equivalent time points obtained from a handled control group, where available. Flies that died within 24 h after treatment were excluded from the analysis.

### 2.3. Bacterial infection

*Serratia marcescens* (ATCC #8100) were grown overnight in LB medium to saturating concentrations. The next day, the culture was diluted in phosphate buffered saline containing 1% food coloring (Brilliant Blue FCF) to an $O.D._{600}$ of 0.03. Three to four days after flies were loaded into monitors, they were removed from the incubator, anesthetized with $CO_2$ and the diluted bacterial solution was manually injected using glass capillary needles (WPI, Inc., #1B100F-4), as described previously (Kuo et al., 2012). The monitors were then returned to the incubator, and flies were recorded for an additional 5–10 days to monitor survival. Survival was determined using the Insomniac 3 software. A fly was considered dead when activity counts remained at zero for at least 24 h.

Sleep measurements in flies excluded those that died within the first 24 h after inoculation. Due to technical difficulty in performing manual injections, handled control groups were not included in these assays in order to minimize the amount of time that monitors were outside the recording chamber. Changes from baseline sleep were therefore calculated as described above, except values were not normalized to a handled control group.

### 2.4. Statistical analyses

Student's unpaired *t*-test was used to compare sleep parameters between indicated groups. A Bonferroni correction was applied to *p*-values where appropriate. One-way ANOVA with Tukey's post-hoc was used to measure differences between three or more groups. A two-way ANOVA was used to evaluate effects of time of heat pulse and genotype on sleep where indicated. Survival analyses were conducted as described previously (Kuo and Williams, 2014a). Briefly, the Kaplan–Meier estimator was used to calculate survival probability and a log rank test was used to evaluate differences between survival curves. All statistical analyses were performed using public-accessed software (PAST; http://folk.uio.no/ohammer/past/) (Hammer et al., 2001).

## 3. Results

### 3.1. Heat stress induces sleep

We first determined the effects of heat stress (HS) on sleep in wild-type animals. *Canton-S* and $w^{1118}$ wild-type flies were placed at 37 °C for 1 h (see Methods) at zeitgeber time (ZT) 18, which is 6 h into the dark phase of a 12 h:12 h light:dark cycle. This time point was chosen based on findings that infection and aseptic injury at ZT18 induce robust effects on sleep (Kuo et al., 2010). The HS flies exhibited increases in sleep relative to handled controls (HC) in the morning hours after HS, a time that coincides with lights-on and a typical period of arousal in handled control or undisturbed flies (Fig. 1A). Specifically, a consistent and robust increase in sleep was observed from ZT 0-6 following HS in both wild type strains (Fig. 1B). Increased sleep was also observed during the second half of the day in $w^{1118}$ flies, from ZT 6-12, albeit to a lesser extent than at ZT 0-6 (Fig. 1B).

We considered the possibility that the increased activity and concomitant sleep loss caused by the elevated temperature at night resulted in a homeostatic sleep rebound response the following morning. To address this concern, we subjected $w^{1118}$ flies to a temperature of 31 °C or 34 °C for 1 h at ZT 18. Both temperatures disturbed sleep as determined across a 4 h period (ZT 16-20; Supplementary Fig. 1). Despite exhibiting similar amounts of lost sleep

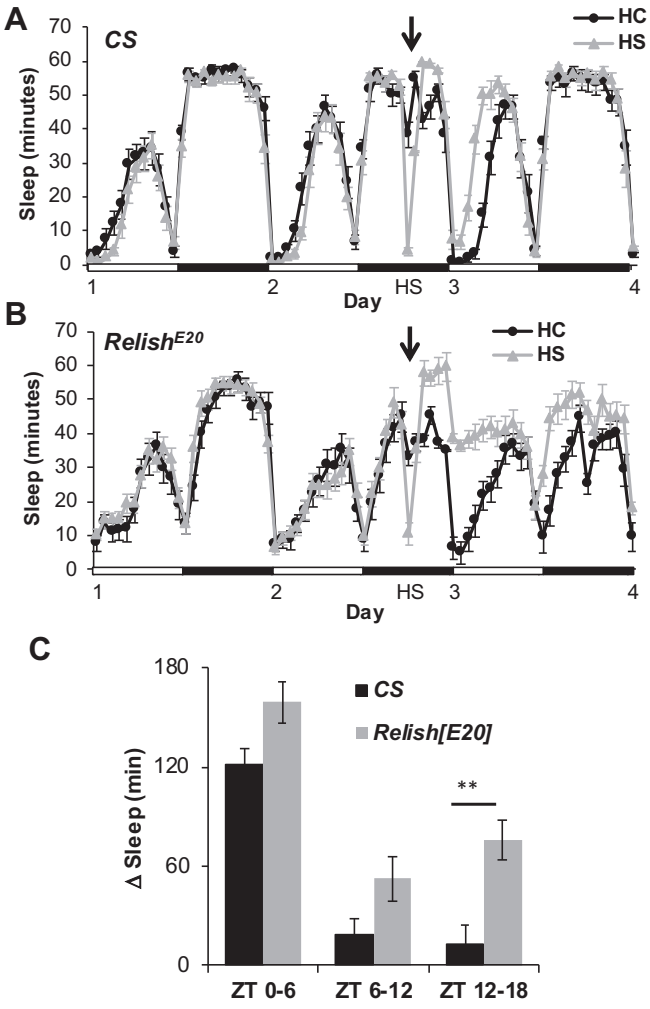


**Fig. 2.** Heat stress induces sleep in $Relish^{E20}$ mutants. Mean ± SEM minutes sleep per hour plotted across 3 days in the assay in (A) *CS* ($n = 16$ flies each for handled control (HC) and heat shock (HS) group) and (B) $Relish^{E20}$ mutants ($n = 24$, 18 flies for HC, HS groups, respectively). (C) Mean ± SEM change in sleep is shown at indicated times on the day following HS at ZT 18 in CS and in $Relish^{E20}$ mutants. $^{**}p < .001$, *t*-test, Bonferroni corrected ($n = 52$ for both *CS* and $Relish^{E20}$).

to HS at 37 °C, flies exposed to the lower temperatures did not sleep more than handled controls during the day after the heat pulse. Specifically, after HS at 31 °C, flies showed a net change of sleep that was less than baseline (−29.6 ± 7.9 min from ZT 0-6, $p < 0.002$, one-way *t*-test, $n = 16$), and no change when flies were exposed to 1 h at 34 °C (−2.1 ± 4.3 min from ZT 0-6, $p = 0.62$, one-way *t*-test, $n = 48$). Therefore, the HS-induced sleep response is due to the stress of the increased temperature and is not (solely) due to the incurred sleep debt.

To evaluate potential time-of-day effects of HS, we compared the effects of HS at ZT18 (during the middle of the night period) to the effects of HS at ZT0 (lights on), ZT6 (during the middle of the day period), and ZT12 (lights off). All four time points elicited significantly increased sleep over the following 24 h in both $w^{1118}$ and *CS* flies (Fig. 1C). A modest but significant time-of-day effect was detected in $w^{1118}$ flies ($p < 0.02$, one-way ANOVA; Fig. 1C). This was attributed to a weaker response when flies were subjected to HS at ZT 12 as compared to that at ZT 18 ($p < 0.01$, Tukey's post-hoc). No significant time-of-day effect was observed in *CS* flies ($p = 0.2$, one-way ANOVA; Fig. 1C). These data indicate that, unlike infection-induced sleep, HS-induced sleep is not strongly dependent on the time-of-day of the heat pulse. However, we noted that the increase in sleep measured across the 24 h was largely

attributed to an increase in daytime sleep (Fig. 1A), leaving open the possibility that the circadian clock may determine the distribution of increased sleep across the day. The variations in responses of $w^{1118}$ flies in comparison to *CS* indicate an influence of the genetic background, as previously described for other sleep measurements (Zimmerman et al., 2012).

Temperature is a known zeitgeber in *Drosophila* (Glaser and Stanewsky, 2007). Even mild and brief heat pulses are capable of producing a phase shift in locomotor activity rhythms in flies in a manner similar to that of a light pulse (Sidote et al., 1998). Although HS was applied at times of day that are not associated with producing significant shifts in activity phase (Dushay et al., 1990), we nonetheless sought to determine whether the HS-induced sleep in the morning is the result of a temporary phase shift. We therefore subjected flies to HS in the absence of a functioning circadian clock, in constant light. Constant light (LL) degrades integral clock genes in flies and renders them arrhythmic (reviewed in (Devlin and Kay, 2001)). In the LL condition, HS increased sleep immediately following the heat pulse (Supplementary Fig. 2). This indicates that a phase shift cannot account for the increase in subsequent morning sleep.

### 3.2. Heat stress induced sleep does not require NFκB

The sleep response to septic/aseptic injury requires the NFκB transcription factor Relish (Kuo et al., 2010, 2012; Kuo and Williams, 2014a), which is central to the *Drosophila* innate immune response (Hedengren et al., 1999). We therefore tested the hypothesis that Relish is generally required for other stress-induced sleep responses including that induced by heat stress. This hypothesis predicts that genetic absence of Relish function would impair HS-induced sleep. We tested this prediction by subjecting $Relish^{E20}$ null mutants to HS at ZT 18. Surprisingly, $Relish^{E20}$ mutants showed a robust induction of sleep in response to HS at ZT 18 (Fig. 2B). In comparison to CS wild-type controls, the increase in sleep extended beyond the daytime, to at least ZT 18 of the following night (Fig. 2C). Therefore, genetic pathways distinct from Relish regulate HS-induced sleep. This indicates that sleep induced by different types of stress is regulated by distinct mechanisms.

### 3.3. FR deficient flies have a reduced sleep response to HS

Because FMRFamide-like peptides encoded by the gene *flp-13* are required for the sleep-like response to heat stress in *C. elegans* (Nelson et al., 2014), we tested whether the *Drosophila* FMRFa receptor, FR, regulates HS-induced sleep in flies. We compared the HS-induced sleep responses of flies containing a transposable element, *minos* (Metaxakis et al., 2005) inserted into the FR gene, $FR^{MB04659}$, to $w^{1118}$ flies, which is the genetic background strain for this mutant (Bellen et al., 2011). The *minos* insertion in $FR^{MB04659}$ flies is located in the coding region, which is contained within a single exon of the *FR* gene and spans nucleotides 3,008,746 to 3,010,395 on the left arm of chromosome 3. This insertion predicts the removal of 447 nucleotides of coding sequence from the 3′ end of the gene, affecting all isoforms of *FR*. The program SMART (Schultz et al., 1998) predicts that this transposon insertion results in a truncated protein containing only four of the seven transmembrane domains and removes the intracellular G-protein coupling domain. Thus, this allele likely represents a null mutation in *FR*.

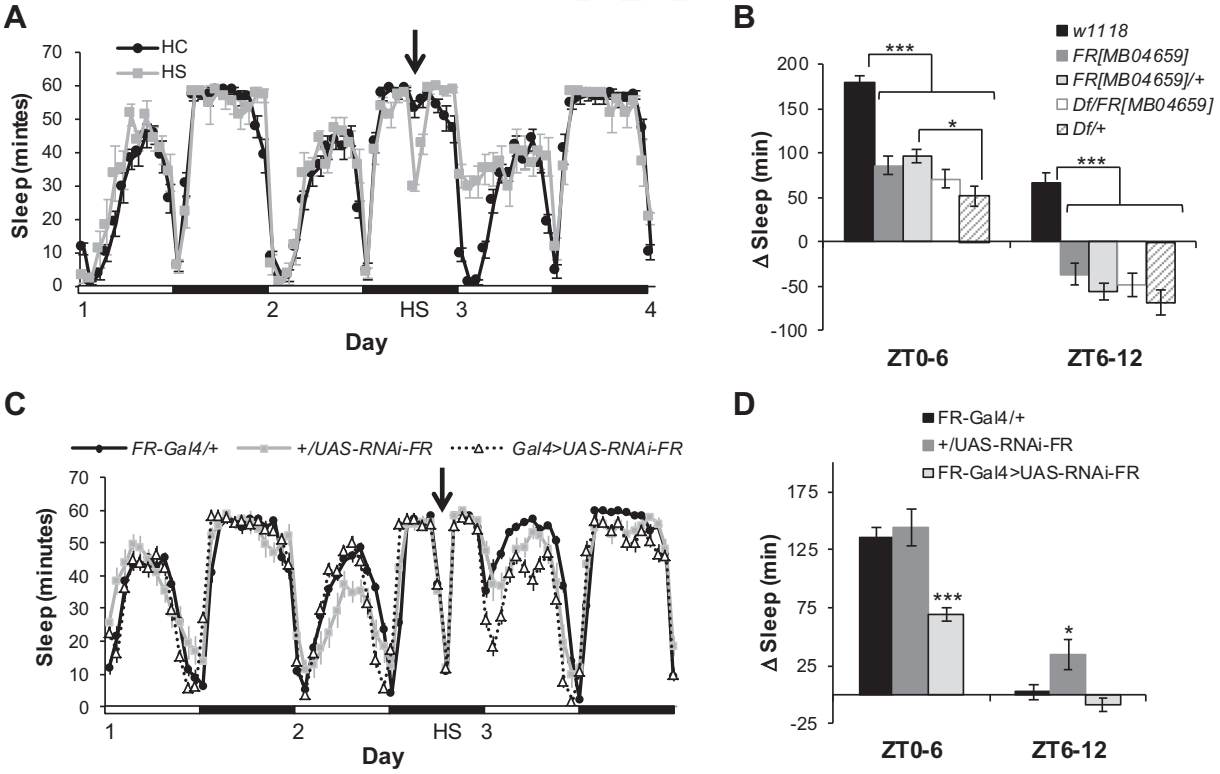


**Fig. 3.** Heat stress-induced sleep is decreased in *FR* mutants. (A) Mean ± SEM minutes sleep per hour plotted across 3 days in the assay for $FR^{MB04659}$ ($n = 16$ for handled control (HC); and 12 for heat shock (HS) groups); (B) Net change in sleep is reported for the day following HS at ZT 18 for indicated genotypes. *FR* mutants show significantly reduced sleep at both time points ($p < .00001$ one-way ANOVA); $^{***}p < .00001$, and $^{*}p < .01$, Tukey's post-hoc; $n = 46$ $w^{1118}$; 30 $FR^{MB04659}$; 31 $FR^{MB04659}$/+; 30 *Df*/$FR^{MB04659}$; and 32 *Df*/+, where *Df* = *Df*(*3L*)*BSC428*. (C) Experiment as described in (A) showing responses of $FR^{MB04659}$/+ (*FR-Gal4*/+, $n = 32$), +/*UAS-RNAi-FR* ($n = 15$) and *FR-Gal4>UAS-RNAi-FR* ($n = 31$) to HS. (D) Change in sleep is plotted for indicated genotypes. One-way ANOVA showed significant effects of genotype for both time points ($p < 0.0001$); $^{***}p < .0005$; $^{*}p < .01$, Tukey's post hoc ($n = 48$, 31, and 63 for *FR-Gal4*/+, +/*UAS-RNAi-FR*, and *FR-Gal4>UAS-RNAi-FR* flies, respectively).

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Homozygous *FR* mutants and $w^{1118}$ wild-type controls were subjected to HS at ZT 18. As compared to $w^{1118}$ controls, HS-induced sleep was significantly reduced in $FR^{MB04659}$ flies (Fig. 3A and B). HS-induced sleep was also reduced in FR mutants when the HS was applied at ZT6 (Supplementary Fig. 3A), suggesting that time-of-day effects are unlikely to explain the FR reduced HS-induced sleep phenotype. There was no difference in baseline daytime or nighttime sleep between genotypes ($p = 0.75$ and 0.46, respectively; *t*-test; $n = 54$ $w^{1118}$ and 61 $FR^{MB04659}$ flies). While daytime waking activity rate was also unchanged ($p = 0.25$), $FR^{MB04659}$ flies showed a modest but significant decrease in average nighttime waking activity (2.07 ± 0.05 activity counts/waking minute in $FR^{MB04659}$ flies versus 2.3 ± 0.07 activity counts/waking minute in $w^{1118}$ flies; $p < 0.01$, *t*-test). Interestingly, waking activity rates increased during the HS in both groups. In $w^{1118}$ flies, waking activity rate remained elevated through the next day, but in $FR^{MB04659}$ flies, it did not (Supplementary Fig. 3C). Together, these data indicate that while $FR^{MB04659}$ flies show normal sleep behavior at baseline, HS-induced behavior is disrupted at multiple levels.

In the course of performing these behavioral experiments, we noted that HS was partially lethal in $FR^{MB04659}$ flies, such that about 25% of flies were killed by HS, whereas survival was nearly 100% in $w^{1118}$ flies (Supplementary Fig. 3B). Therefore, $FR^{MB04659}$ flies are defective both for HS-induced behaviors and for survival after heat stress exposure.

To determine whether the effect of the *Minos*-element insertion was attributed to a lesion in the *FR* chromosomal locus, $FR^{MB04659}$ flies were crossed with those carrying a small chromosomal deficiency that included the FR locus, *Df(3L)BSC428*. $FR^{MB04659}$ and *Df(3L)BSC428* flies were also crossed to the $w^{1118}$ background to obtain heterozygous offspring. Both $FR^{MB04659}$/+ and *Df(3L)BSC428*/+ heterozygous mutants showed significantly reduced sleep responses to HS at ZT 18 as compared to $w^{1118}$ wild type controls (Fig. 3B), indicating that the phenotype associated with both mutants is dominant. HS-induced sleep in the $FR^{MB04659}$/*Df(3L)BSC428* transheterozygotes was no different from the heterozygous mutants; the lack of an additive effect of the two mutants indicates that the phenotypes are likely attributed to lesions of the *FR* locus. HS-induced sleep was reduced in all genotypes as compared to the $w^{1118}$ controls (Fig. 3B).

The *Minos* element also contains a sequence that codes for GAL4, a transcription factor from yeast that can be used to activate a UAS promoter when expressed as a transgene in *Drosophila* (Brand and Perrimon, 1993). Expression of GAL4 is controlled by the gene in which *Minos* inserted, in this case the gene *FR*. We made use of the GAL4/UAS system to knock down FR using RNA interference specifically in cells expressing FR. We crossed $FR^{MB04659}$ flies with those carrying a *UAS-RNAi* transgene directed against *FR*, $FMRFaR^{lF01879}$ (*UAS-RNAi-FR*). Both parent lines were also crossed to $w^{1118}$ background controls, and all offspring were subjected to HS at ZT 18. $FR^{MB04659}$>*UAS-RNAi-FR* flies showed significantly reduced responses to HS as compared to both parent lines (Fig. 3C and D). Together, these findings support a role of FR in stress-induced sleep.

FR is activated by FMRFa neuropeptides (Cazzamali and Grimmelikhuijzen, 2002; Johnson et al., 2003; Meeusen et al., 2002), which are related to nematode FLP-13 neuropeptides. We therefore evaluated responses of flies carrying a p-element inserted into the *FMRFa* gene, $FMRFa^{KG01300}$. Homozygous $FMRFa^{KG01300}$ mutants and *y,w* controls, which are the genetic background for these mutants (Bellen et al., 2004), were subjected to HS at ZT 18. *y,w* flies responded to HS by increasing sleep from ZT 0-4. Thus, *y,w* flies were less sensitive than $w^{1118}$ and *CS* flies to heat-shock, consistent with the notion that sleep measurements in *Drosophila* are sensitive to genetic background (Zimmerman et al., 2012). Nonetheless, the $FMRFa^{KG01300}$ mutant flies showed significantly reduced sleep responses in comparison to the *y,w* controls (Fig. 4). These results support the hypothesis that FMRFa peptides are important for HS-induced sleep.

### 3.4. FR deficient flies are defective in infection-induced sleep and are susceptible to bacterial infection

The experiments we performed with *Relish* mutants described above indicate that HS-induced sleep is controlled by a mechanism that is partially distinct from that induced by bacterial infection. We aimed to determine whether FMRFa/FR signaling is a pathway unique to HS-induced sleep or whether it is part of a mechanism common to infection- and HS-induced sleep. To test this possibility, $FR^{MB04659}$ flies and $w^{1118}$ control flies were subjected to infection with *S. marcescens* at ZT 18. $w^{1118}$ flies showed a robust sleep response to infection at ZT 0-4, the morning after inoculation. In contrast, the sleep response to infection in $FR^{MB04659}$ mutant flies was significantly reduced (Fig. 5A and B). Survival time during infection was also severely reduced in $FR^{MB04659}$ flies (Fig. 5C), consistent with prior experiments showing a beneficial effect of infection-induced sleep (Kuo and Williams, 2014a). Both $FR^{MB04659}$ and $w^{1118}$ control flies exhibited nearly 100% survival when subjected to aseptic injury (Fig. 5D), indicating that injury alone did not cause the survival outcomes during infection. These findings suggest that while the signaling events that induce sleep are different between infection and heat stress, these events converge on FR to produce a robust sleep response, and promote survival.

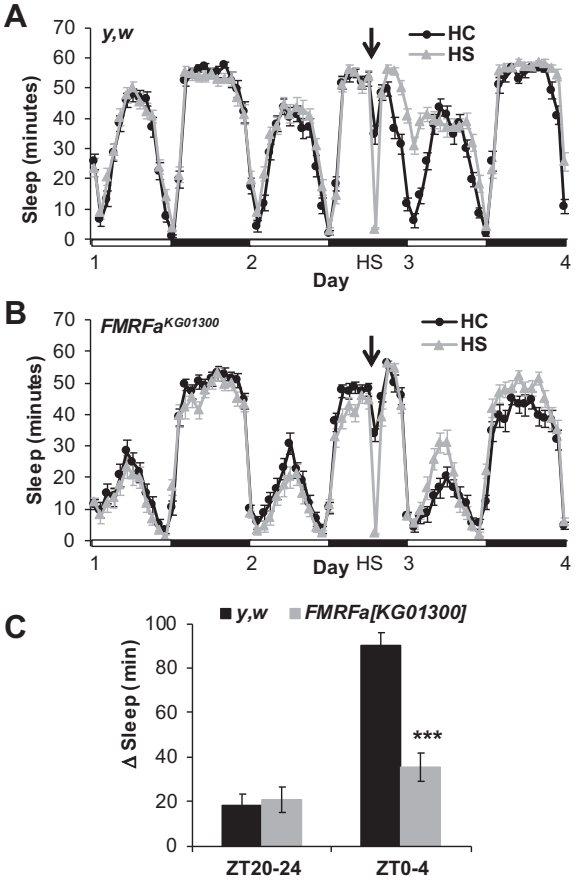


**Fig. 4.** Heat stress-induced sleep is decreased in FMRFa mutants. Mean ± SEM minutes sleep per hour plotted across 3 days in (A) *y,w* controls, and (B) $FMRFa^{KG03100}$ mutants ($n = 32$ flies for each group). (C) Net change in sleep following HS at ZT 18 (***$p < .0001$, *t*-test, Bonferroni corrected; $n = 61$ *y,w* and 64 $FMRFa^{KG03100}$ flies).

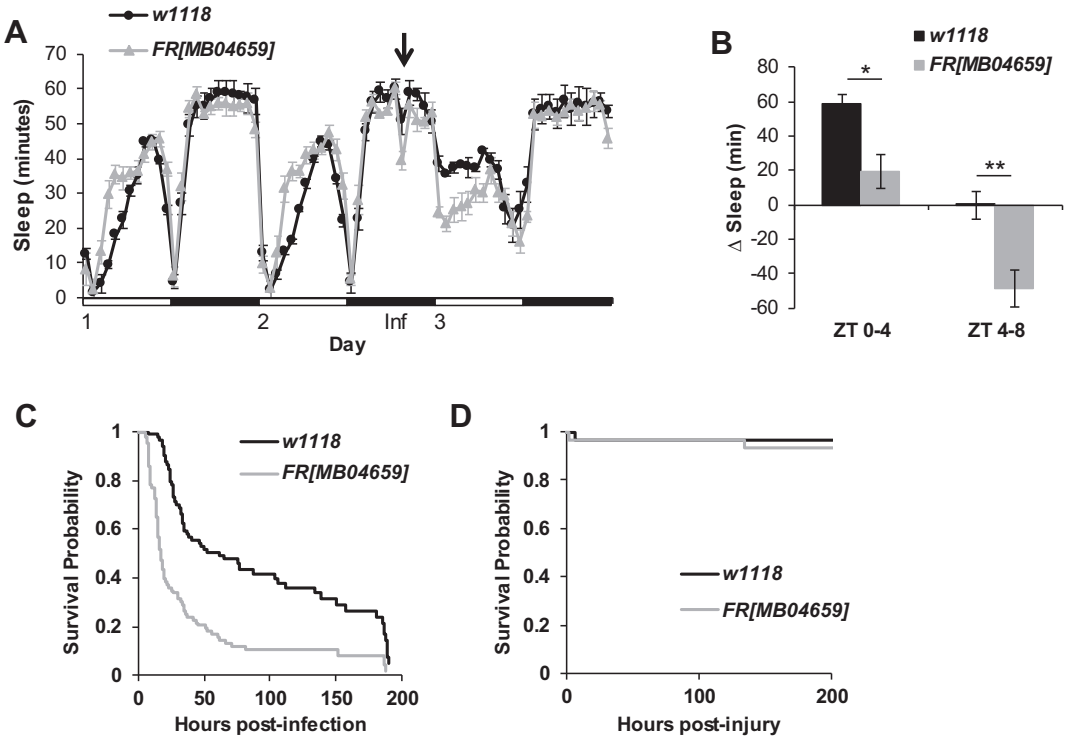


**Fig. 5.** Post-infection sleep and survival is reduced in FR mutants. (A) Mean ± SEM minutes sleep per hour; "Inf" and arrow indicate time of infection with *S. marcescens* ($n$ = 28, 23 for $w^{1118}$ and $FR^{MB04659}$ flies, respectively. B. Net change in sleep per 4 h is plotted for indicated times the day after infection. $^{**}p < 0.001$, $^{*}p < 0.02$, $t$-test, Bonferroni corrected; $n$ = 70 and 30 $w^{1118}$ and $FR^{MB04659}$ flies, respectively. Kaplan–Meier plot of survival probability following (C) infection ($p < 0.00001$, log rank test, $n$ = 83 $w^{1118}$, and 85 $FR^{MB04659}$ flies) and (D) aseptic injury ($p = 0.6936$, log rank test, $n$ = 32 both groups).

## 4. Discussion

Sleep is influenced by multiple environmental and behavioral factors, such as social experience (Ganguly-Fitzgerald et al., 2006), light stimuli (Harbison and Sehgal, 2009; Shang et al., 2011), copulation (Isaac et al., 2010), diet (Catterson et al., 2010; Keene et al., 2010; Linford et al., 2012; Thimgan et al., 2010; Yamazaki et al., 2012), and septic injury (Kuo et al., 2010; Shirasu-Hiza et al., 2007). Here we show a conserved response to heat stress, where similar to nematodes (Hill et al., 2014; Nelson et al., 2014), flies respond to heat shock with an increase in sleep predominantly in the first half of the daytime. The magnitude of heat stress-induced sleep was sensitive to genetic background, and unlike sleep induced by immune challenge (Kuo et al., 2010), it was not sensitive to the time-of-day of the heat pulse. *Relish* null mutants, which are defective for infection-induced sleep (Kuo et al., 2010), are not defective for HS-induced sleep; surprisingly, they show an exaggerated response to heat shock. The heat stress-induced sleep is regulated by FMRFa peptides and their receptor FR. *FR* is also required for the normal sleep response to an infection as well as for survival during an infection. Together, these findings indicate that while different stress modalities are transduced by distinct molecular mechanisms, these signaling events converge onto FR to promote sleep.

Temperature is a known zeitgeber in flies, such that they entrain to a diurnal temperature cycle, even in constant light (Glaser and Stanewsky, 2005; Yoshii et al., 2005). Mild heat pulses also induce phase shifts in circadian activity rhythms by suppressing expression of the clock gene, PERIOD (PER) (Sidote et al., 1998). The phase responses to heat pulses are also similar to those induced by light (Sidote et al., 1998). These findings indicate that temperature is an important environmental input to the circadian clock. However, the morning increase in sleep induced by a 37 °C heat pulse is unlikely to be attributed to a phase shift in activity rhythms for the following reasons. First, heat pulse causes an enhanced sleep response even when delivered at ZT6, a time when there is a negligible circadian phase response to light (Dushay et al., 1990). Second, our experiments are performed under light:-dark conditions, which results in the daily resetting of activity rhythms. Third, mild heat pulses (31 and 34 °C), which is sufficient to produce phase shifts in both molecular and behavioral rhythms (Sidote et al., 1998), does not increase morning sleep. Finally, HS in the absence of the clock, under constant light conditions, elicits an enhanced sleep response immediately following the heat pulse. Together these findings indicate that the daytime increase in sleep following HS cannot be attributed to a shift in activity phase. Although the amplitude of the sleep response is not strongly altered by the time-of-day of the heat pulse, the response is primarily restricted to daytime hours. Thus a role of the clock cannot entirely be ruled out and requires further study.

*FR* mutants show reduced survival both in response to bacterial infection and heat stress, consistent with the notion that recovery sleep provides a beneficial function to the animal. This notion is supported by our prior observations that increasing infection-induced sleep in $Relish^{E20}$ mutants improves survival (Kuo and Williams, 2014a). Whether rescuing stress-induced sleep in *FR* mutants produces a similar enhancement in survival requires further study. In any case, these data support the notion that sleep is an adaptive process that promotes survival.

Along with the current finding that FMRFa signaling promotes stress-induced sleep, neuropeptides are emerging as important signaling molecules with diverse roles in sleep and circadian rhythms. For example, the neuropeptide pigment dispersing factor (PDF) is involved in both arousal (Parisky et al., 2008; Sheeba et al.,



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2008) and clock output mechanisms (Renn et al., 1999). Recent work has shown sleep promoting roles of SIFamide (Park et al., 2014) and sNPF (Shang et al., 2013) secreted from pars intercerebralis and small ventrolateral clock cells, respectively. The precise role of sNPF, which, Like FMRFa, is a member of the FaRP family (Nassel and Winther, 2010), remains uncertain since it has also been reported to serve a wake-promoting role (Chen et al., 2013). Previous studies have shown that FMRFamide signaling is involved in regulating larval escape responses to stress, as indicated by decreased activity of *FR* mutants during stressful stimuli (Kiss et al., 2013; Klose et al., 2010). As previously described for larvae, our experiments reveal the adult $FR^{MB04659}$ mutants have a reduced waking activity during and after heat stress. Thus the activity phenotype associated with this mutant does not correlate with the sleep phenotype. We propose that an optimal escape response to environmental stress enables animals to quickly migrate to a safer environment that would facilitate sleep. In support of this hypothesis, video observations have shown that even in the absence of environmental stress, fruit flies move to preferred areas in the recording chamber to sleep (Hendricks et al., 2000; Zimmerman et al., 2008).

While infection-induced sleep clearly requires the NFκB *Relish* (Kuo et al., 2010), it is likely that heat shock proteins (Hsp) mediate responses to heat stress. Hsp's have been reported to function in sleep homeostasis, although a specific role of these molecules in sleep is not well understood. Sleep deprivation increases expression of Hsp's in mammals (Terao et al., 2003) as well as in fruit flies (Shaw et al., 2002; Williams et al., 2007; Zimmerman et al., 2006). Flies deficient in expression of the heat shock protein Hsp83 show exaggerated responses to sleep deprivation and to have poor survival in response to a prolonged period of sleep deprivation (Shaw et al., 2002). The current study suggests that Hsp's may function as early signaling events that promote sleep, and that the neuropeptide, FMRFa, is responsive to this signal. Alternatively, both sleep deprivation and heat shock induced sleep may allow cells to restore protein homeostasis through the action of Hsps.

Cellular stress induced quiescence occurs also in *C. elegans* adult animals (Hill et al., 2014). This quiescence has the sleep-like properties of cessation of feeding and locomotion, reduced responsiveness to weak stimuli, and rapid reversibility in response to strong stimuli (Hill et al., 2014). The current study, together with work in *C. elegans*, supports a model of a conserved molecular mechanism for stress-induced sleep. The ALA neuron, which is receptive to epidermal growth factor (EGF) (Van Buskirk and Sternberg, 2007), is necessary for heat-stress induced sleep (Hill et al., 2014). EGF receptor signaling also promotes sleep in flies (Foltenyi et al., 2007) and mammals (Kramer et al., 2001). Whether it is also required for stress-induced sleep in insect and mammalian species will be an important topic for future study. Nelson et al. (2014) further showed that the ALA neuron secretes quiescence promoting FLP-13 peptides, which share an RFamide motif with the *Drosophila* FMRFa peptides. In flies, an EGF receptor ligand is secreted from the brain region called the pars intercerebralis (PI) and targets receptors in the tritocerebrum to promote sleep (Foltenyi et al., 2007). Whether these cells also contain FMRFa peptides as part of a sleep promoting circuit analogous to worms is unknown. Our results indicate that FMRFa peptides acts on the G-protein coupled receptor FR to promote sleep. FR is most closely related to a group of paralogous *C. elegans* GPCRs encoded by the genes *frpr-4, -6, -11, -12, -16*, and *-18* (M. Nelson and D. Raizen, unpublished observations). It remains to be determined whether or not these receptors function in nematode stress-induced quiescence. Future experiments performed in both *Drosophila* and *C. elegans* can test this specific predictions made by our model.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2014.12.028.

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